OXA1P
ENHANCED PROTEIN SECRETION

FIELD OF THE INVENTION

The present invention provides methods and compositions for the efficient and enhanced secretion of a protein of interest from a host cell. In specific, proteins are secreted through the Sec-dependent pathway, involving the *spoIIIJ* and/or *yqjG* gene product(s). In some embodiments, expression of the *spoIIIJ* and/or *yqjG* gene product(s) is modulated by a promoter operably linked to the gene.

BACKGROUND OF THE INVENTION

In recent years, not only interesting similarities, but also striking differences between protein transport pathways in eubacteria, archaea, eukaryotes, and eukaryotic organelles have been documented (Pohlschröder *et al.*, Cell 91:563-566 [1997]; Dalbey and Robinson, Trends Biochem. Sci., 24:17-22 [1999]; and Dalbey and Kuhn, Ann. Rev. Cell. Dev. Biol., 16:51-87 [2000]). Insights into the extent of conservation and divergence in these pathways were provided due to the availability of many complete genome sequences. Unfortunately, the biological significance of such insights has often proven difficult, to test as the majority of organisms with sequenced genomes are poorly amenable to biochemical or genetic approaches. In this respect, the Gram-positive eubacterium *Bacillus subtilis*, the complete genome sequence of which was published by Kunst *et al.* (Kunst *et al.*, Nature 390:249-356 [1997]), has been a very useful exception, due to this organism's natural system for genetic transformation and its large capacity for the secretion of proteins directly into the growth medium (*See*, Tjalsma *et al.*, Microbiol. Mol. Biol. Rev., 64:515-547 [2000a]). Nonetheless, much remains unknown regarding the biosynthetic pathways of *Bacillus*.

The functional genomic approach to dissect the protein secretion process in *B. subtilis* has yielded a number of remarkable surprises. These surprises include striking differences in the composition of the general secretion (Sec) and twin-arginine translocation (Tat) pathways for the transport of secretory pre-proteins across the membranes of *B. subtilis* and *E. coli* (Bolhuis *et al.*, J. Biol. Chem.,

273:21217-21224 [1998]; Bolhuis *et al.*, J. Biol. Chem., 274:24531-24538 [1999a]; Jongbloed *et al.*, J. Biol. Chem., 275:41350-41357 [2000]; Robinson and Bolhuis, Nat. Rev. Mol. Cell. Biol., 2:350-356 [2001]; and van Wely *et al.*, Microbiol., 146:2573-2581 [2000]).

In contrast to *Escherichia coli* (*See*, Fekkes and Driessen, Microbiol. Mol. Biol. Rev., 63:161-173 [1999]), the Sec-dependent translocation machinery of *B. subtilis* lacks a SecB component (van Wely *et al.*, J. Bacteriol., 181:1786-1792 [2000]). Moreover, the *B. subtilis* SecDF component, which is present as a natural fusion protein, is merely required to optimize the efficiency of protein translocation under conditions of protein hyper-secretion at gram per litre levels (Bolhuis *et al.*, [1998], *supra*), while the separate SecD and SecF proteins of *E. coli* are very important both for protein export and cell viability (Pogliano and Beckwith, EMBO J., 13:554-561 [1994]). In addition, in contrast to the twin-arginine translocation (Tat) machinery of *E. coli* that consists of the unique TatB and TatC components and the paralogous TatA and TatE components (*See e.g.*, Robinson and Bolhuis, Nat. Rev. Mol. Cell. Biol., 2:350-356 [2001]), the Tat machinery of *B. subtilis* lacks distinguishable TatA/E and TatB components, while two paralogous TatC proteins with distinct functions are present (Jongbloed *et al.*, [2000], *supra*).

Translocated pre-proteins of *B. subtilis* with Sec-type or twin-arginine signal peptides have been shown to be subject to processing by the largest number of type I signal peptidases (SPases) known in various organisms. In addition to five chromosomally-encoded SPases (SipS, SipT, SipU, SipV, and SipW [Tjalsma *et al.*, Genes Dev., 12:2318-2331 (1998)]), some *B. subtilis* strains contain plasmid-encoded SPases (Meijer *et al.*, Mol. Microbiol., 17:621-631 [1995]). Furthermore, SipW was the first known eubacterial SPase of a type that is mainly encountered in archaea and the eukaryotic endoplasmic reticular membrane (Tjalsma *et al.*, [1998], *supra*; and Tjalsma *et al.*, J. Biol. Chem., 275:25102-25108 [2000b]).

Another finding was that the unique type II SPase (Lsp) of *B. subtilis* (Prágai *et al.*, Microbiol., 143:1327-1333 [1997]), which specifically catalyzes the maturation of lipid-modified pre-proteins is required for the secretion of non-lipoproteins, such as α-amylase, chitosanase, and lipase (Tjalsma *et al.*, J. Biol. Chem., 274:1698-1707 [1999]; and Antelmann *et al.*, Genome Res., 11:1484-1502 [2001]). When the

negative effect of an *lsp* mutation on non-lipoprotein secretion was first observed for the α-amylase AmyQ, it was largely attributed to a possible malfunctioning of the lipoprotein PrsA, which is essential for the proper folding of various translocated proteins, such as AmyQ.

In addition, the original view that Gram-positive eubacteria would lack thiol-disulfide oxidoreductases for the formation of disulfide bonds in secretory proteins was disproved with the identification of three Bdb proteins (Bolhuis *et al.*, J. Biol. Chem., 274:24531-24538 [1999b]). Indeed, any lesson learned regarding protein secretion and the relative contribution of homologs from Gram-negative bacteria is not necessarily be relevant to Gram-positive microorganisms. Thus, there remains a need in the art to provide means to assess and identify secretory proteins in Gram-positive organisms such as *Bacillus*.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for the efficient secretion of proteins from Gram-positive microorganisms.

In some preferred embodiments, the present invention provides DNA constructs for the inducible expression of the *spoIIIJ* and/or *yqjG* gene(s). In particularly preferred embodiments, the *spoIIIJ* and/or *yqjG* gene(s) is/are operably linked to a promoter sequence. In some embodiments, the promoter is inducible, while in other embodiments the promoter is constitutive.

In some embodiments, a second DNA sequence is inserted into the host cell. In preferred embodiments, the second sequence encodes a Sec-dependent signal sequence that is inked or fused to a protein of interest. In some embodiments, the protein of interest is homologous, while in other embodiments, it is heterologous to the host cell.

The present invention further provides methods for modulating Sec-dependent protein secretion comprising the steps of: introducing a *spoIIIJ* gene linked to an inducible promoter into a *Bacillus* cell; and modulating the expression of the *spoIIIJ* gene by varying the level of induction of the inducible promoter. In some embodiments, the inducible promoter is the P*spac* promoter. In alternative embodiments, the methods of inhibiting sporulation in a *Bacillus* cell, comprise a

mutation of the *spoIIIJ* gene wherein the mutation results in the formation of an inactive gene product. In additional embodiments, the present invention provides purified DNA molecules comprising an inducible promoter operatively linked to the *spoIIIJ* gene.

The present invention also provides methods for modulating the secretion of a protein of interest, comprising the steps of: forming a first DNA molecule encoding a chimeric protein comprising a Sec-dependent secretion signal peptide; forming a second DNA molecule encoding an inducible promoter operably linked to the *spoIIIJ* gene; transforming a host cell with the DNA molecule of steps the previous steps; and growing the host cell under conditions wherein the protein of interest is expressed at the desired level. In some embodiments, the host cell is grown under conditions wherein the inducible promoter is induced. In still further embodiments, the protein of interest is expressed at low level.

The present invention further provides methods of modulating Sec-dependent protein secretion comprising the steps of: introducing a *yqjG* gene linked to an inducible promoter into a *Bacillus* cell; and modulating the expression of the *yqjG* gene by varying the level of induction of the inducible promoter. In some embodiments, the inducible promoter is the P*spac* promoter.

The present invention also provides methods of inhibiting sporulation in a *Bacillus* cell, wherein the methods comprise a mutation of the *spoIIIJ* gene wherein the mutation results in the formation of an inactive gene product. In still further embodiments, the present invention provides purified DNA molecules comprising an inducible promoter operatively linked to the *yqjG* gene.

The present invention further provides methods for modulating the secretion of a protein of interest, comprising the steps of: forming a first DNA molecule encoding a chimeric protein comprising a Sec-dependent secretion signal peptide; forming a second DNA molecule encoding an inducible promoter operably linked to the *yqjG* gene; transforming a host cell with the DNA molecule of steps the previous steps; and growing the host cell under conditions wherein the protein of interest is expressed at the desired level. In some embodiments, the host cell is grown under conditions wherein the inducible promoter is induced. In still further embodiments, the protein of interest is expressed at low level.

The present invention also provides methods of modulating Sec-dependent protein secretion comprising the steps of: providing a *Bacillus* cell comprising *spoIIIJ* and *yqjG* genes linked to an endogenous high expression promoter; and modulating the expression of the *spoIIIJ* and *yqjG* genes by varying the level of induction of the promoter. In some embodiments, the promoter is the P*spac* promoter.

Other objects, features and advantages of the present invention will become apparent from the present Specification. It should be understood, however, that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the scope and spirit of the invention will become apparent to one skilled in the art from this detailed description.

DESCRIPTION OF THE FIGURES

Figure 1, Panel A provides a sequence comparison showing the conservation of Oxa1p(-like) proteins in *B. subtilis*, *E. coli and* mitochondria of the yeast *S. cerevisiae*. In this Panel, the amino acid sequences of SpoIIIJ and YqjG, and the partial sequences of YidC and Oxa1p, comprising the conserved part as depicted in Panel B are shown. Identical residues are indicated in bold. The conserved transmembrane segments I-V (marked in gray shading) were predicted as described by Sipos and von Heijne (Sipos and von Heijne, Eur. J. Biochem., 213:1333-1340 [1993]). The putative SPase II cleavage sites (lipoboxes) in SpoIIIJ and YqjG are underlined. Notably, SpoIIIJ/YqjG orthologues with putative lipoprotein signal peptides have been found in several Gram-positive eubacteria (during the development of the present invention, SpoIIIJ/YQJG orthologues with putative lipoprotein signal peptides were identified in *B. anthracis*, *B. halodurans*, *B. stearothermophilus*, *Lactococcus lactis*, and *Staphylococcus aureus*). In this Panel, the numbers refer to the position of amino acids in the corresponding protein sequence.

Figure 1, Panel B, provides the predicted membrane topologies of SpoIIIJ and YqjG of *B. subtilis,* YidC of *E. coli,* and Oxa1p of *S. cerevisiae*. Only the membrane topology of YidC has been verified experimentally (Saaf *et al.*, J. Biol.

Chem., 273:30415-30418 [1998]). Notably, the YidC protein has a large amino-terminal loop located in the periplasm that is absent from SpoIIIJ, YqjG and Oxa1p. The abbreviations used in this Figure are: C, carboxyl-terminus; the cytoplasmic, cell wall, periplasmic, matrix, or inter membrane space (IMS) sides of the membranes, and the "conserved" parts of the Oxa1p(-like) proteins are indicated. In addition, as shown in this Figure, the amino-termini of SpoIIIJ and YqjG are most likely lipid-modified.

Figure 2 shows the construction of *spoIIIJ* and/or *yqjG* mutant strains of *B. subtilis*. As indicated, *B. subtilis* Δ*spoIIIJ* and *B. subtilis* Δ*yqjG* were respectively, constructed by the single cross-over integration of pMutin-Δ*spoIIIJ* and pMutin-Δ*yqjG* into the chromosome *B. subtilis* 168. In these strains, the respective *spoIIIJ* or *yqjG* genes are disrupted while the *lacZ* gene of pMutin2 is placed under the transcriptional control of the promoter regions of these genes. *B. subtilis* Δ*yqjG*-Tc was constructed by replacement of the 5' part of the *yqjG* gene with a tetracycline resistance marker by double cross-over recombination. *B. subtilis* Δ*yqjG*-I*spoIIIJ* was constructed by integration of pMutin-I*spoIIIJ* into the *spoIIIJ* region of *B. subtilis* Δ*yqjG*-Tc. Using this approach, the *spoIIIJ* gene was placed under the control of the (IPTG)-dependent P*spac* promoter. Notably, growth and viability of the latter strain is dependent on the presence of IPTG. The relative positions of open reading frames in the *spoIIIJ* and *yqjG* regions are shown. PCR-amplified DNA fragments that were used to direct the integration of pMutin2 into the *B. subtilis* chromosome are indicated with black bars. The abbreviations (including restriction sites relevant for the construction) used in this Figure are: B, *Bam*HI; dco, double cross-over, E, *Eco*RI; H, *Hin*dIII; *jag*, gene of unknown function specifying a predicted cytoplasmic protein; P, *Pst*I. Ori pBR322, replication functions of pBR322; Emr, erythromycin resistance marker; sco, single cross-over; T1T2, transcriptional terminators on pMutin2; *spoIIIJ'*, 3' truncated *spoIIIJ* gene; '*spoIIIJ*, 5' truncated *spoIIIJ* gene; *yqjG'*, 3' truncated *yqjG* gene; and '*yqjG*, 5' truncated *yqjG* gene.

Figure 3 depicts properties of *spoIIIJ* and/or *yqjG* mutant strains of *B. subtilis*. In Panel A, IPTG-dependent growth of *B. subtilis* Δ*yqjG*-I*spoIIIJ* on plates incubated at 15°C and 37°C is shown. Individual colonies of *B. subtilis* Δ*yqjG*-I*spoIIIJ* were transferred to fresh TY-agar plates containing 1000, 500, 100, 50, or 0 nM IPTG.

Next, the plates were incubated overnight at 37°C (upper panel), or for 5 days at 15°C (lower panel).

Figure 3, Panel B shows the IPTG-dependent growth of *B. subtilis* Δ*yqjG*-I*spoIIIJ*. Overnight cultures of *B. subtilis* 168 (parental; Δ), *B. subtilis* Δ*spoIIIJ* (▲), *B. subtilis* Δ*yqjG* (○) and *B. subtilis* Δ*yqjG*I*spoIIIJ* (■), grown in TY medium at 37°C, were washed and diluted 20-fold in fresh TY medium without IPTG and incubated at 37 °C. In addition, *B. subtilis* Δ*yqjG*-I*spoIIIJ* was diluted in fresh TY medium containing 50 (□) or 500 (●) nM IPTG. Zero time (t=0) indicates the transition point between the exponential and post-exponential growth phases.

Figure 3, Panel C provides the transcription profiles of the *spoIIIJ* and *yqjG* genes. Time courses of the transcription of the *spoIIIJ-lacZ* and *yqjG-lacZ* gene fusions in *B. subtilis* Δ*spoIIIJ* (○) and *B. subtilis* Δ*yqjG* (■) were determined in cells growing at 37°C in TY medium. β-galactosidase activities were determined in Units per OD_{600}. Zero time (t=0) indicates the transition point between the exponential and post-exponential growth phases.

Figure 4 provides data showing that SpoIIIJ and YqjG are required for efficient protein secretion.

Figure 4, Panel A provides results for precultures of *B. subtilis* Δ*yqjG*-I*spoIIIJ* and the parental strain *B. subtilis* 168, both transformed with plasmid pLip2031 (specifying LipA), pPSPPhoA5 (specifying PhoA), or pKTH10 (specifying AmyQ), prepared by overnight growth at 37°C in TY medium containing 500 nM IPTG. Next, cells were washed with fresh TY medium without IPTG, diluted 20-fold in fresh TY medium containing 500 nM, 50 nM or no (0) IPTG, and incubated for 3 hours at 37°C before sampling for SDS-PAGE and Western blotting. Specific antibodies were used to detect the cellular (pre-)LipA, PhoA, or (pre-)AmyQ levels (upper panels), and the levels of secreted LipA, PhoA, or AmyQ in the growth medium (lower panels). The positions of pre-LipA and pre-AmyQ (○), mature LipA, PhoA and AmyQ (●), or degradation products of PhoA (*) are indicated.

Figure 4, Panel B provides data from experiments in which processing of pre-AmyQ in *B. subtilis* Δ*yqjG*-I*spoIIIJ* was analyzed by pulse-chase labeling at 37°C in S7 medium with (500 nM; upper panel) or without (0) IPTG (lower panel), subsequent immunoprecipitation, SDS-PAGE, and fluorography. Cells were labeled

with [35S]-methionine for 1 min prior to chase with excess non-radioactive methionine. Samples were withdrawn after the chase at the times indicated. Since the incorporation of label into (pre-)AmyQ cannot be stopped instantaneously by the addition of non-radioactive methionine, samples withdrawn at t=0, and t=1 contain lower amounts of labeled AmyQ than the sample withdrawn at t=5. The positions of pre-AmyQ (o) and mature AmyQ (•) are indicated.

Figure 4, Panel C shows the translocation of pre-AmyQ-PSBT in SpoIIIJ-depleted cells lacking YqjG. To analyse pre-AmyQ translocation, cells of *B. subtilis* Δ*yqjG*-I*spoIIIJ*, *B. subtilis* D*secDF* (positive control) and the parental strain *B. subtilis* 168 (negative control) were transformed with plasmid pKTH10-BT (specifying AmyQ-PSBT), and grown as described for Panel A. Cellular (biotinylated) AmyQ-PSBT was visualized by SDS-PAGE and Western blotting using AmyQ-specific antibodies (upper panel), or a streptavidin-HRP conjugate (lower panel). Precursor (o) and mature (•) forms of AmyQ-PSBT are indicated.

Figure 5 provides data showing that SpoIIIJ and YqjG are required for efficient post- translocationally folding and release of secretory proteins. Processing and release of pre-AmyQ (Panel A), pre-pro-PhoA (Panel B), and pre-LipA (Panel C) were analyzed in *B. subtilis* Δ*yqjG*-I*spoIIIJ* and the parental strain *B. subtilis* 168 by pulse-chase labeling at 37°C in S7 medium, subsequent cell fractionation (protoplasts, cell wall and medium), immunoprecipitation, SDS-PAGE, and fluorography. Cells were labeled with [^{35}S]-methionine for 1 min prior to chase with excess non-radioactive methionine. Samples were withdrawn at 1, 2, and 10 min after the chase. Cells and medium were separated by centrifugation, and protoplasts were subsequently prepared as described in the Examples. Finally, the cell wall and protoplast fractions were separated by centrifugation. The positions of pre-AmyQ and pre-pro-PhoA (o), degradation products of (pro-) PhoA and AmyQ (*) and mature AmyQ, PhoA and LipA (•) are indicated.

Figure 6 provides illustrates that SpoIIIJ and YqjG are of minor importance for membrane protein biogenesis. Cells of *B. subtilis* Δ*yqjG*-I*spoIIIJ* xSecDF-Myc (Δ*yqjG*-I*spoIIIJ*) and the control strain *B. subtilis* xSecDF-Myc (parental), were grown overnight at 37°C in TY medium containing 500 nM IPTG. Next, cells were washed with fresh TY medium without IPTG, diluted 20-fold in fresh TY medium containing

1% xylose (production SecDF-Myc) and 500 nM, 50 nM or no (0) IPTG, and incubated at 37°C for 3 hours. Samples for SDS-PAGE and Western blotting were prepared from cells and specific antibodies were used to detect the cellular levels of SecDF-Myc, SipS, PrsA, FtsH, CtaC, and QoxA. The positions of the native proteins (•) and their degradation products (*) are indicated. Notably, FtsH-derived degradation products (FtsH, lower panel) were only visible after prolonged fluorography. Putative membrane topologies of these proteins are depicted (N, amino terminus; C, carboxyl-terminus; in, cytoplasmic side of the membrane; out, cell wall-exposed side of the membrane). In addition, this Figure indicates that the amino-termini of PrsA, CtaC and QoxA are lipid-modified.

Figure 7 shows results of protease mapping of membrane proteins in SpoIIIJ-depleted cells lacking YqjG. To analyse the insertion of the membrane proteins SecDF-Myc, SipS, PrsA, FtsH, CtaC and QoxA, cells of *B. subtilis* Δ*yqjG*-I*spoIIIJ* xSecDF-Myc (Δ*yqjG*-I*spoIIIJ*) and the control strain *B. subtilis* xSecDF-Myc (parental), were grown overnight at 37°C in TY medium containing 500 nM IPTG. Cells were washed with fresh TY medium without IPTG, diluted 20-fold in fresh TY medium without IPTG, and incubated at 37°C for 3 hours. Next, the production of SecDF-Myc was induced by the addition of 1% xylose 15 min prior to protoplasting. Protoplasts were incubated for 30 min without further additions, in the presence of trypsin (1 mg/ml), or trypsin and Triton X-100 (1%). This procedure was performed in parallel with cells of *B. subtilis* Δ*yqjG*-I*spoIIIJ* and *B. subtilis* 168 (parental), both containing pKTH10 for production of AmyQ. Samples were used for SDS-PAGE, Western blotting, and specific antibodies were used to detect SecDF-Myc, SipS, PrsA, FtsH, CtaC, QoxA, AmyQ or GroEL. The positions of intact proteins and pre-AmyQ (•), degradation products due to the incubation with trypsin (*), mature AmyQ (○), and trypsin-resistant fragments (►) are indicated.

Figure 8 provides data showing pulse-chase/protease mapping of membrane proteins in SpoIIIJ-depleted cells lacking YqjG. To analyse the kinetics of SipS, PrsA and QoxA insertion, cells of *B. subtilis* Δ*yqjG*-I*spoIIIJ* (Δ*yqjG*-I*spoIIIJ*) and the control strain *B. subtilis* 168 (parental) were grown at 37°C in S7 medium, and labeled with [35S]-methionine for 1 min followed by a chase of 1 min with excess non-radioactive methionine. Cells were collected by centrifugation, and protoplast

were subsequently prepared as described in the Examples. Protoplasts were incubated for 30 min without further additions, in the presence of trypsin (1 mg/ml), or trypsin and Triton X-100 (1%) prior to immunoprecipitation, SDS-PAGE, and fluorography. Immunoprecipitation with anti-GroEL antibodies was used to check protoplast integrity. The positions of intact proteins (•) and trypsin-resistant fragments (►) are indicated.

DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for the efficient secretion of proteins from Gram-positive microorganisms. All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

Definitions

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.,* DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

As used herein, the term "host cell" refers to a cell that has the capacity to act as a host and expression vehicle for an expression cassette according to the invention. In one embodiment, the host cell is a Gram-positive microorganism. In a preferred embodiment according to the present invention, "host cell" refers to members of the genus *Bacillus*. As used herein, the genus *Bacillus* includes all members known to those of skill in the art, including but not limited to *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus, B. clausii, and B. thuringiensis*.

As used herein, the term "polypeptide" refers to a compound made up of amino acid residues linked by peptide bonds. The term "protein" as used herein may be synonymous with the term "polypeptide" or may refer, in addition, to a complex of two or more polypeptides.

As used herein, the terms "protein of interest" and "polypeptide of interest" refer to the protein to be expressed and secreted by the host cell. The protein of interest may be any protein that up until now has been considered for expression in prokaryotes. The protein of interest may be either homologous or heterologous to the host.

As used herein, the terms "chimeric polypeptide" and "fusion polypeptide" are used interchangeably in reference to a protein that comprises at least two separate and distinct regions that may or may not originate from the same protein. For example, a signal peptide linked to the protein of interest wherein the signal peptide is not normally associated with the protein of interest would be termed a chimeric polypeptide or chimeric protein.

As used herein, the terms "signal peptide" and "signal sequence" refer to an amino-terminal extension on a protein to be secreted. Nearly all secreted proteins use an amino-terminal protein extension which plays a crucial role in the targeting to and translocation of precursor proteins across the membrane and which is proteolytically removed by a signal peptidase during or immediately following membrane transfer. In preferred embodiments of the present invention, the signal sequence is the sec-dependent signal peptides derived from *Bacillus*.

As used herein, the term "enhanced" refers to improved production of

proteins of interest. In preferred embodiments, the present invention provides enhanced (*i.e.*, improved) production and secretion of a protein of interest. In these embodiments, the "enhanced" production is improved as compared to the normal levels of production by the host (*e.g.*, wild-type cells). Thus, for heterologous proteins, basically any expression is enhanced, as the cells normally do not produce the protein.

As used herein, the terms "isolated" and "purified" refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

As used herein, the term "heterologous protein" refers to a protein or polypeptide that does not naturally occur in a host cell. Examples of heterologous proteins include enzymes such as hydrolases including proteases, cellulases, amylases, other carbohydrases, and lipases; isomerases such as racemases, epimerases, tautomerases, or mutases; transferases, kinases and phosphatases, hormones, growth factors, cytokines, antibodies and the like.

A "heterologous" nucleic acid construct or sequence has a portion of the sequence that is not native to the cell in which it is expressed. "Heterologous," with respect to a control sequence refers to a control sequence (*i.e.* promoter or enhancer) that does not function in nature to regulate the same gene the expression of which it is currently regulating. Generally, heterologous nucleic acid sequences are not endogenous to the cell or part of the genome in which they are present, and have been added to the cell, by infection, transfection, microinjection, electroporation, or the like. In some embodiments, "heterologous" nucleic acid constructs contain a control sequence/DNA coding sequence combination that is the same as, or different from a control sequence/DNA coding sequence combination found in the native cell.

As used herein, the term "homologous protein" refers to a protein or polypeptide native or naturally occurring in a host cell. The present invention encompasses host cells producing the homologous protein via recombinant DNA technology. The present invention further encompasses a host cell which may have one or more deletions or one or more interruptions of the nucleic acid encoding the naturally occurring homologous protein or proteins, such as, for example, a

protease, and having nucleic acid encoding the homologous protein or proteins re-introduced in a recombinant form (*i.e.*, in an expression cassette). In other embodiments, the host cell produces the homologous protein.

As used herein, the term "nucleic acid molecule" includes RNA, DNA and cDNA molecules. It will be understood that, as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given protein may be produced.

As used herein, the term "vector" refers to a nucleic acid construct designed for transfer between different host cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

As used herein, the terms "expression cassette" and "expression vector" refer to a nucleic acid construct generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter.

As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a cloning vector, and which forms an extrachromosomal self-replicating genetic element in many bacteria and some eukaryotes.

As used herein, the term "selectable marker-encoding nucleotide sequence" refers to a nucleotide sequence which is capable of expression in the host cells and where expression of the selectable marker confers to cells containing the expressed gene the ability to grow in the presence of a corresponding selective agent or lack of an essential nutrient.

As used herein, the term "promoter" refers to a nucleic acid sequence that functions to direct transcription of a downstream gene. In preferred embodiments, the promoter is appropriate to the host cell in which the target gene is being

expressed. The promoter, together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences") is necessary to express a given gene. In general, the transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

As used herein, "chimeric gene" and "heterologous nucleic acid construct" refer to a non-native gene (*i.e.*, one that has been introduced into a host) that may be composed of parts of different genes, including regulatory elements. A chimeric gene construct for transformation of a host cell is typically composed of a transcriptional regulatory region (promoter) operably linked to a heterologous protein coding sequence, or, in a selectable marker chimeric gene, to a selectable marker gene encoding a protein conferring antibiotic resistance or other selectable properties to transformed cells. A typical chimeric gene of the present invention, for transformation into a host cell, includes a transcriptional regulatory region that is constitutive or inducible, a signal peptide coding sequence, a protein coding sequence, and a terminator sequence. In some embodiments, chimeric gene constructs also include a second DNA sequence encoding a signal peptide if secretion of the target protein is desired.

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA encoding a secretory leader, i.e., a signal peptide, is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the term "gene" means the segment of DNA involved in

producing a polypeptide chain, that may or may not include regions preceding and following the coding region (*e.g.* 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences), as well as intervening sequences (introns) between individual coding segments (exons).

In some embodiments, the gene encodes therapeutically significant proteins or peptides, such as growth factors, cytokines, ligands, receptors and inhibitors, as well as vaccines and antibodies. The gene may encode commercially important industrial proteins or peptides, such as enzymes (*e.g.*, proteases, carbohydrases such as amylases and glucoamylases, cellulases, oxidases and lipases). However, it is not intended that the present invention be limited to any particular enzyme or protein. In some embodiments, the gene of interest is a naturally-occurring gene, a mutated gene or a synthetic gene.

A nucleic acid sequence is considered to be "selectively hybridizable" to a reference nucleic acid sequence if the two sequences specifically hybridize to one another under moderate to high stringency hybridization and wash conditions. Hybridization conditions are based on the melting temperature I of the nucleic acid binding complex or probe. For example, "maximum stringency" typically occurs at about Tm-5°C (5° below the Tm of the probe); "high stringency" at about 5-10°C below the Tm; "intermediate stringency" at about 10-20°C below the Tm of the probe; and "low stringency" at about 20-25°C below the Tm. Functionally, maximum stringency conditions may be used to identify sequences having strict identity or near-strict identity with the hybridization probe; while high stringency conditions are used to identify sequences having about 80% or more sequence identity with the probe.

Moderate and high stringency hybridization conditions are well known in the art. An example of high stringency conditions includes hybridization at about 42°C in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured carrier DNA followed by washing two times in 2X SSC and 0.5% SDS at room temperature and two additional times in 0.1X SSC and 0.5% SDS at 42°C.

As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid sequence or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express

genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention. "Recombination, "recombining," or generating a "recombined" nucleic acid is generally the assembly of two or more nucleic acid fragments wherein the assembly gives rise to a chimeric gene.

As used herein, the terms "transformed," "stably transformed," and "transgenic" used in reference to a cell means the cell has a non-native (heterologous) nucleic acid sequence integrated into its genome or as an episomal plasmid that is maintained through two or more generations.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

As used herein, the term "introduced" used in the context of inserting a nucleic acid sequence into a cell, means "transfection," "transformation," or "transduction," and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell where the nucleic acid sequence may be incorporated into the genome of the cell (*e.g.*, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (for example, transfected mRNA).

In one embodiment, present invention finds use in enhancing the secretion of any protein of interest that may be made to be secreted via the Sec-dependent secretion pathway. Any protein of interest that may be fused to a Sec-dependent signal peptide by recombinant DNA methods finds use in the present invention.

In particularly preferred embodiments of the present invention, the host cell is rendered capable of enhanced secretion of a protein of interest. In some embodiments, the protein of interest is endogenous, while in alternative embodiments, it is heterologous. In further embodiments, the protein of interest is a chimeric protein, in that the native protein of interest is fused to a Sec-dependent signal sequence. In additional embodiments, the host cell is also transformed with a DNA construct encoding the *spoIIIJ* gene. In some preferred embodiments, the *spoIIIJ* gene is operably linked to a promoter. In some embodiments, the promoter

is constitutive, while in other embodiments, it is inducible. One preferred promoter is the P*spac*.

It is contemplated that by varying the level of induction of an inducible promoter that it may be possible to modulate the expression of the *spoIIIJ* gene product and thereby modulate the secretion of the protein of interest. It is also contemplated that a host cell deficient in the *spoIIIJ* gene may have impaired protein secretion.

In preferred embodiments of the present invention, the host cell is a Gram-positive cell. In particularly preferred embodiments, the Gram-positive cell is a member of the genus *Bacillus*.

DETAILED DESCRIPTION OF THE INVENTION

Prior to the development of the present invention, an unexplained observation was that *B. subtilis* cells lacking SPase II accumulated significantly increased levels of pre-AmyQ (Tjalsma *et al.*, [1999], *supra*). As the latter effect was never observed in *prsA* mutant strains (Kontinen and Sarvas, Mol. Microbiol., 8:727-737 [1993]; and Jacobs *et al.*, Mol. Microbiol., 8:957-966 [1993]), it was concluded that the increased pre-AmyQ accumulation in *lsp* mutant strains was due to the malfunction of one or more lipoproteins other than PrsA. This resulted in the development of the present invention in which lipoproteins of *B. subtilis* with previously unknown roles in protein secretion are provided.

During the development of the present invention, the amino acid sequences of all 114 (predicted) lipoproteins of *B. subtilis* (Tjalsma *et al.*, [1999], *supra*) were used for similarity searches in public databases. Strikingly, the predicted lipoproteins SpoIIIJ and YqjG both showed significant similarity to the Oxa1 protein of yeast mitochondria (*See*, Figure 1). This inner membrane protein has been implicated in the export of the amino- and carboxyl-termini of the mitochondrially-encoded precursor of cytochrome c oxidase subunit II (pre-CoxII) from the mitochondrial matrix (Hell *et al.*, FEBS Lett., 418:367-370 [1997]; and Hell *et al.*, Proc. Natl. Acad. Sci. USA 95:2250-2255 [1998]). In addition, it was recently shown that the *E. coli* orthologue of Oxa1p, denoted YidC, is associated with the Sec translocase (Scotti *et al.*, EMBO J., 19:542-549 [2000]). However, Samuelson and

co-workers (Samuelson *et al.*, Nature 406:637-641 [2000]) demonstrated that YidC of *E. coli* is of major importance for the biogenesis of several membrane proteins, whereas the export of secretory proteins was hardly impaired in YidC-depleted cells.

Despite the differences in the roles of SpoIIIJ and YqjG in protein expression among various organisms, the present invention provides the identification of roles of SpoIIIJ and YqjG in protein secretion and/or membrane protein biogenesis. Indeed, results obtained during the development of the present invention show that SpoIIIJ and YqjG are important for post-translocational stages in protein secretion. Consistent with an important role in secretion, the presence of at least one of these two Oxa1p orthologues is essential for cell growth. It is noted that under SpoIIIJ- and YqjG-limiting conditions that strongly affect protein secretion, the stability of various membrane proteins is not, or only very mildly, affected. Also, while SpoIIIJ is essential for sporulation (Errington *et al.*, J. Gen. Microbiol., 138:2609-2618 [1992]), its paralogue YqjG is not involved in this developmental process. Taken together, these data indicate that SpoIIIJ and YqjG have acquired functions that are at least partly different from those of other members of the Oxa1 family.

Recently, the *yidC* gene of *E. coli*, specifying a SpoIIIJ/YqjG orthologue (Figure 1), was shown to be essential for cell viability (*See*, Samuelson *et al., supra*). In contrast, the *spoIIIJ* gene of *B. subtilis* is not essential for growth. In fact, *spoIIIJ* was originally identified as the gene containing the *spo-87* mutation that blocks sporulation of *B. subtilis* cells at stage III, after the completion of prespore engulfment (Errington *et al., supra*). To search for possible functions of the YqjG protein, a *yqjG* disruption strain was constructed with the integration vector pMutin2 (*See*, Figure 2). The fact that *B. subtilis* Δ*yqjG* could be obtained showed that YqjG, like SpoIIIJ, is not essential for growth (*See*, Figure 3, Panel A). Notably, disruption of the *yqjG* gene did not detectably affect sporulation, while a Δ*spoIIIJ* control strain constructed with pMutin2 was unable to develop viable spores (data not shown). Thus, as discussed in greater detail in the Experimental section, YqjG has no essential function in the sporulation process, in contrast to SpoIIIJ.

As indicated above, proteins homologous to Oxa1p are conserved in eubacteria and eukaryotic organelles, where they appear to have important

functions in protein transport and membrane assembly. In fact, SpoIIIJ of *B. subtilis* was the first protein of this family to which a function was assigned. Errington and co-workers (Errington *et al., supra*) reported that a *spoIIIJ* mutation blocks sporulation of *B. subtilis* cells at stage III, after the completion of forespore engulfment. It was thought that this protein was involved in a signal transduction pathway, coupling gene expression in the forespore to concomitant events in the mother cell. However, the exact function of SpoIIIJ in sporulation was not unraveled.

During the development of the present invention, it was determined that the synthesis of either SpoIIIJ, or its paralogue YqjG, is required for growth of the Gram-positive eubacterium *B. subtilis*. Furthermore, as discussed in greater detail below, the secretion of the mature AmyQ, PhoA and LipA proteins into the growth medium was strongly impaired under conditions of SpoIIIJ/YqjG-limitation. Notably, the reduction in AmyQ levels in the medium was not paralleled by reduced rates of pre-AmyQ translocation and processing, or the cellular accumulation of pre-AmyQ, as previously documented for *secDF, tepA* or *sip* mutant strains with translocation defects (Bolhuis *et al.*, [1998], *supra*; Tjalsma *et al.*, [1998], *supra*; Bolhuis *et al.*, [1999a], *supra*; and Tjalsma *et al.*, J. Biol. Chem., 272:25983-25992 [1997]). Pulse-chase experiments combined with cell fractionation showed that degradation of AmyQ and PhoA occurs in the membrane-cell wall interface soon after the proteins have left the translocation channel (*See*, Figure 7). Thus, the secretion defect observed with SpoIIIJ-depleted cells lacking YqjG occurs in the post-translocational stages that involve the folding of secretory proteins into their active and protease-resistant conformation. The latter is of particular importance because the extracytoplasmic side of the membrane, the cell wall and the growth medium of *B. subtilis* are highly proteolytic (Tjalsma *et al.*, [2000a], *supra*; and Antelmann *et al.*, Genome Res., 11:1484-1502 [2001]). In a wild-type cell, it is contemplated that SpoIIIJ and YqjG facilitate the folding of translocated proteins in at least two different ways. However, it is not intended that the present invention be limited to any particular mechanism. Nonetheless, it is conceivable that SpoIIIJ and

YqjG have a direct role in the correct folding of mature proteins shortly after their translocation across the membrane, similar to the essential function proposed for the folding catalyst PrsA (Tjalsma *et al.*, [1999], *supra*; and Kontinen *et al., supra*). Alternatively, it is contemplated that SpoIIIJ and YqjG have an indirect role in protein secretion by modulating the activity of folding catalysts, such as PrsA. The fact that SpoIIIJ-depleted cells lacking YqjG do not accumulate increased levels of the precursor form of AmyQ, as previously observed in cells lacking SPase II, indicates that the typical lipoproteins SpoIIIJ and YqjG are not responsible for the accumulation of pre-AmyQ in SPase II mutant cells. Thus, unprocessed lipoproteins other than SpoIIIJ and YqjG must be responsible for the latter phenomenon, unless the secretion defects caused by impaired pre-SpoIIIJ and pre-YqjG processing in the absence of SPase II are different from those caused by SpoIIIJ/YqjG limitation. However, as indicated above, it is not intended that the present invention be limited to any particular or specific mechanism.

In recent years, the Oxa1p protein of yeast mitochondria was shown to be required for the
processing of mitochondrially-encoded precursors (Bauer *et al.*, Mol. Gen. Genet., 245:272-278 [1994]), the export of amino- and carboxyl-termini from pre-Cox II synthesized in the mitochondrial matrix (Hell *et al.*, [1997], *supra*; and Hell *et al.*, [1998], *supra*) and the insertion of transmembrane domains into the mitochondrial inner membrane in a pairwise fashion (Herrmann *et al.*, EMBO J., 16:2217-2226 [1997]). Taking into consideration the fact that yeast mitochondria completely lack Sec components, and that the Oxa1p orthologue in chloroplasts (Albino III) is required for the Sec-independent integration of the light-harvesting chlorophyll-binding protein into the thylakoid membrane (Moore *et al.*, J. Biol. Chem., 275:1529-1532 [2000]), it is contemplated that members of the Oxa1p family represent the key components of a novel pathway for protein export or membrane protein assembly (Stuart and Neupert, Nature 406:575-577 [2000]). Consistent with this view, YidC of *E. coli* was shown to facilitate the Sec-independent insertion of certain membrane proteins, such as the M13 procoat, being of minor importance for the export of Sec-dependent pre-proteins (Samuelson *et al., supra*). Nevertheless, YidC, was also shown to be associated with the Sec machinery, indicating that this protein has a

more general role in membrane protein biogenesis in *E. coli*, for example by catalyzing the exit of membrane proteins from the Sec translocase (Scotti *et al., supra*; Samuelson *et al., supra*; and Houben *et al.*, FEBS Lett., 476:229-233 [2000]). Indications for such a lateral movement were obtained by Urbanus and co-workers (Urbanus *et al.*, EMBO Rep., 2:524-529 [2001]), who demonstrated a sequential interaction of the membrane protein FtsQ with SecY and YidC. Recent studies by Houben *et al.* (Houben *et al.*, J. Biol. Chem., 277:35880-35886 [2002]) showed that YidC has the ability to contact a transmembrane domain very early during biogenesis, when it is not even fully exposed outside the ribosome. Thus, it is contemplated that YidC has a role in both the reception and lipid partitioning of transmembrane segments. The results obtained during the development of the present invention indicate that the essential function of SpoIIIJ and YqjG in *B. subtilis* relates to a general role in protein secretion. However, it is not intended that the present invention be limited to any particular or specific mechanism.

The results obtained during the development of the present invention support the view that these proteins are not involved in the actual membrane insertion and translocation of secretory pre-proteins. Furthermore, the stability of the membrane proteins FtsH and CtaC is affected under conditions of SpoIIIJ/YqjG limitation. However, pulse-chase/protease mapping experiments showed that the insertion kinetics of PrsA SipS and QoxA were not significantly affected after chase of a 1-min under these conditions. It should be noted that the membrane topology of QoxA is similar to that of Lep of *E. coli*, of which the insertion was significantly affected after a 2-min chase in YidC-depleted cells (*See*, Samuelson *et al., supra*). Although, pulse-chase experiments could not be performed with SecDF-Myc, CtaC, and FtsH to monitor the kinetics of membrane insertion under conditions of SpoIIIJ/YqjG-depletion, it is contemplated that the defect on the post-translocational folding of secretory proteins is the most prominent effect of SpoIIIJ/YqjG depletion in *B. subtilis*. Thus, results obtained during the development of the present invention, taken together with the well-documented requirement of Oxa1p and YidC for membrane protein biogenesis, indicate that SpoIIIJ and YqjG have specific roles in membrane protein biogenesis in *B. subtilis*. Thus, it is contemplated that different

orthologous members of the Oxa1 protein family have acquired different species or genus-specific functions in Sec-dependent and Sec-independent membrane protein biogenesis and protein secretion. Interestingly, not only orthologous Oxa1-like proteins, but also Oxa1-like paralogues within one organism have acquired (partly) distinct functions, as evidenced by the present observation that SpoIIIJ, but not YqjG, is required for spore development. Similarly, only one of the two Oxa1p orthologues of *Schizosaccharomyces pombe* is essential for respiration (Bonnefoy *et al.*, Mol. Microbiol., 35:1135-1145 [2000]). As only minor differences in the timing of transcription have been observed for the genes specifying the paralogous Oxa1p proteins of *B. subtilis* (See, Figure 3, Panel C) and *S. pombe* (*See*, Bonnefoy *et al.*, [2000], *supra*), the differences in function of these protein pairs are, most likely, based on differences in their primary structures.

As SpoIIIJ and YqjG are important for post-translocational protein folding steps in the secretion process, it is contemplated that SpoIIIJ is specifically required for the folding of certain, translocated sporulation factors. The importance of protein transport for sporulation is underscored by the observations that SecA, and the type I SPases, SipT and SipV, are required for this process, as indicated by results obtained during the development of the present invention and data included in various reports (Bonnefoy *et al.*, [2000], supra; Jiang *et al.*, J. Bacteriol., 182:303-310 [2000]). In addition, it is contemplated that SpoIIIJ is involved in the insertion of specific membrane proteins that are essential for the sporulation process after stage III. It is further contemplated that these membrane proteins are involved in the communication between the forespore and the mother cell. However, as indicated above, it is not intended that the present invention be limited to any particular or specific mechanism.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); µM (micromolar); N (Normal); mol (moles); mmol (millimoles); µmol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); kg

(kilograms); µg (micrograms); L (liters); ml (milliliters); µl (microliters); cm (centimeters); mm (millimeters); µm (micrometers); nm (nanometers); ° C. (degrees Centigrade); h (hours); min (minutes); sec (seconds); msec (milliseconds); Ci (Curies) mCi (milliCuries); µCi (microCuries); TLC (thin layer achromatography); Ts (tosyl); Bn (benzyl); Ph (phenyl); Ms (mesyl); Et (ethyl), Me (methyl); SPase (signal peptidase); Ap (ampicillin); Cm (chloramphenicol); Em (erythromycin); HRP (horseradish peroxidase); IPTG (isopropyl-β-D-thiogalacto-pyranoside); Km (kanamycin); OD (optical density); PAGE (polyacrylamide gel electrophoresis); SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis); PCR (polymerase chain reaction); RBS (ribosome binding site); Tc (tetracycline); TY (tryptone/yeast extract); Roche (Roche Molecular Biochemicals, Roche Diagnostics, Indianapolis, IN); Millipore (Millipore Corp., Bedford, MA); Amersham (Amersham Biosciences, Piscataway, NJ); Clontech (BD Clontech, Palo Alto, CA); Sigma (Sigma Aldrich, St. Louis, MO).

EXAMPLE 1

Plasmids, Bacterial Strains, Media and Assay Systems

Table I lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). Minimal medium for *B. subtilis* was prepared as described by Tjalsma *et al.* ([1998], *supra*). Schaeffer's sporulation medium (SSM) was prepared as described by Schaeffer *et al.* (Schaeffer *et al.*, Proc. Natl. Acad Sci. USA 54:704-711 [1965]). When required, medium for *E. coli* was supplemented with kanamycin (Km; 40 µg/ml), ampicillin (Ap; 50µg/ml), or erythromycin (Em; 100 µg/ml); media for *B. subtilis* were supplemented with chloramphenicol (Cm; 5 µg/ml), Km (10 µg/ml), tetracycline (Tc; 6 µg/ml), or Em (1µg/ml).

The pMutin2 plasmid was described by Vagner *et al.*, (Vagner *et al.*, Microbiol., 144:3097-3104 [1998]), while the pUK21 plasmid was described by Vieria and Messing (Vieria and Messing, Gene 100:189-194 [1990]), pLip2031 was described by Dartois *et al.* (Dartois *et al.*, Appl. Environ. Microbiol., 60:1670-1673 [1994]), pKTH10 was described by Palva (Palva, Gene 19:81-87 [1982]), and

pKTH10-BT was described by Tjalsma *et al.*, (Tjalsma *et al.*, [1998], *supra*).

The *E. coli* MC1061 strain was described by Wertman *et al* (Wertman *et al.*, Genen., 49:253-262 [1986]), while the *B. subtilis* 168 strain was described by Kunst *et al.* (Kunst *et al.*, Nature 390:249-256 [1997]), the *B. subtilis ΔsecDF* and xSecDF-Myc strains were described by Bolhuis *et al.* (Bolhuis *et al.*, J. Biol. Chem., 273:21217-21224 [1998]).

Table I. Plasmids and Bacterial Strains

	Relevant Properties
Plasmids	
pMutin2	pBR322-based integration vector for *B. subtilis*; contains a multiple cloning site downstream of the P*spac* promoter, and a promoter-less *lacZ* gene preceded by the RBS of the *spoVG* gene; Ap^r; Em^r
pMutin-Δ*spoIIIJ*	As pMutin2, contains an internal fragment of the *B. subtilis spoIIIJ* gene
pMutin-I*spoIIIJ*	As pMutin2, contains the 5' part of the *B. subtilis spoIIIJ* gene
pMutin-Δ*yqjG*	As pMutin2, contains an internal fragment of the *B. subtilis yqjG* gene
pUK21	Cloning vector; 2.8 kb; Km^r
pUK*yqjG*-Tc	pUK21 derivative for the disruption of *yqjG*; Km^r ; Tc^r
pPSPPhoA5	Plasmid carrying *E. coli phoA* gene fused to the prepro region of the lipase gene from *Staphylococcus hyicus*; Cm^r
pLip2031	Encodes LipA from *B. subtilis* Km^r
pKTH10	Encodes AmyQ of *Bacillus amyloliquefaciens*; Km^r
pKTH10-BT	As pKTH10, encodes the AmyQ-PSBT fusion protein
Strains	
E. coli	
MC1061	F⁻ ; *araD*139 ; Δ (*ara-leu*)7696 ; Δ (*lac*)X74 ; *galU* ; *galK* ; *hsdR2* ; *mcrA* ; *mcrB1* ; *rspL*
B. subtilis	
168	*trpC2*
Δ*spoIIIJ*	Derivative of 168; contains an integrated copy of plasmid pMutin2 in the *spoIIIJ* gene; *spoIIIJ-lacZ*, Em^r
Δ*yqjG*	Derivative of 168; contains an integrated copy of plasmid pMutin2 in the *yqjG* gene; *yqjG-lacZ*, Em^r
yqjG-Tc	Derivative of 168; 5' part of the *yqjG* gene is replaced with a phosphatases resistance marker, using pUK*yqjG*–Tc, by double

	Relevant Properties
	cross-over recombination; *yqjG::Tc*, Tc^r
Δ*yqjG-lspoIIIJ*	Derivative of *B. subtilis* 168 *yqjG*-Tc; contains an integrated copy of plasmid pMutin2 in the *spoIIIJ* region; *Pspac-spoIIIJ*, *spoIIIJ-lacZ*, Em^r; IPTG-inducible *spoIIIJ* transcription; Tc^r; Em^r
Δ*secDF*	Originally referred to as MIF; derivative of *B. subtilis* 168; contains an integrated copy of plasmid pMutin2 in the *secDF* gene; *secDF-lacZ*, Em^r
xSecDF-Myc	Derivative of *B. subtilis* 168; *amyE::xylA-secDFmyc*; Cm^r; also referred to as XDF-Myc

Assay for Spore Development

The efficiency of sporulation was determined by overnight growth in SSM medium, killing of cells with 0.1 volume chloroform, and subsequent plating.

Beta-Galactosidase Activity Assay

Overnight cultures were diluted 100-fold in fresh medium and samples were taken at hourly intervals for optical density (OD) readings and β-galactosidase activity determinations. The assays and the calculations of β-galactosidase units (expressed as units per OD_{600}) were carried out as known in the art (*See e.g.*, Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, New York [1982]).

Western Blot Analysis and Immunodetection

Western blotting was performed as known in the art (*See e.g.*, Kyhse-Andersen, J. Biochem. Biophys. Meth., 10:203-209 [1984]). After separation by SDS-PAGE, proteins were transferred to Immobilon-PVDF membranes (Millipore). To detect LipA, PhoA, AmyQ(-PSBT), carboxyl-terminally Myc-tagged SecDF, SipS, PrsA, FtsH, CtaC or QoxA, *B. subtilis* cells were separated from the growth medium. Cells were resuspended in lysis buffer (20 mM potassium phosphate, pH 7.5; 15 mM $MgCl_2$; 20% sucrose; 0.5 mg/ml lysozyme) and incubated for 15 min at 37°C. Next, 1 volume of SDS sample buffer (100 mM Tris-HCl [pH 6]; 4% SDS; 10% 2-mercaptoethanol; 30% glycerol; 0.005% bromophenolblue; and 1% Triton X-100) was added and the incubation was prolonged for 15 min at 37°C in the presence of

Complete™ protease inhibitors (Roche). In general, proteins were visualized with the ECL detection system, using rabbit/mouse sera and horseradish peroxidase (HRP)-anti-rabbit/mouse-IgG conjugates (Amersham). Carboxyl-terminally Myc-tagged SecDF was visualized with monoclonal c-Myc antibodies (Clontech), and biotinylated AmyQ-PSBT was visualized with a streptavidin-HRP conjugate (Amersham).

Protein Labeling, Immunoprecipitation, SDS-PAGE and Fluorography

B. subtilis Δ*yqjG*-l*spoIIIJ* was grown overnight in S7 medium (*See*, van Dijl *et al.*, J. Gen. Microbiol., 137:2073-2083 [1991a]) supplemented with 500 nM IPTG, Em (1 µg/ml) and Tc (6 µg/ml). Cells were washed with fresh S7 medium without IPTG and diluted 1:10 in fresh S7 medium containing Em (1 µg/ml), in the presence or absence of IPTG. After 2 hours of growth, cells were resuspended in methionine and cysteine-free S7 (S7 starvation) medium with or without IPTG and grown for another hour prior to pulse-chase labeling. Immunoprecipitation, SDS-PAGE and fluorography were performed as known in the art (*See*, van Dijl *et al.*, Mol. Gen. Genet., 227:40-48 [1991b]; and van Dijl *et al.*, [1991a], *supra*).

Protease Accessibility

Protoplasts were prepared from late exponentially growing cells of *B. subtilis*. Cells were resuspended in protoplast buffer (20 mM potassium phosphate, pH 7.5; 15 mM $MgCl_2$; 20% sucrose) and incubated for 30 min with 1 mg/ml lysozyme (37C). Protoplasts were collected by centrifugation, resuspended in fresh protoplast buffer and incubated at 37°C in the presence of 1 mg/ml trypsin (Sigma) for 30 min. The reaction was terminated by the addition of CompleteTM protease inhibitors (Roche) and protoplasts were used for SDS-PAGE and Western-blotting. In parallel, protoplasts were incubated without trypsin, or in the presence of trypsin and 1% Triton X-100.

EXAMPLE 2
DNA Techniques

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as known in the art (*See e.g.*, Sambrook *et al.* Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York [1989]). Enzymes were obtained from Roche. *B. subtilis* was transformed as known in the art (*See*, Tjalsma *et al.*, [1998], supra). PCR was carried out with Pwo DNA polymerase (Roche), as known in the art (See, van Dijl *et al.* J. Biol. Chem., 270:3611-3618 [1995]). The BLAST algorithm (*See*, Altschul *et al.*, Nucl. Acids Res., 25:3389-3402 [1997]) was used for protein comparisons in GenBank.

To construct *B. subtilis* Δ*spoIIIJ*, an internal fragment of the *spoIIIJ* gene was amplified with the primers *spoIIIJ*-1 (5'- GAG AAT TCG ACG GGA GAT AAC TAC GGG C -3'; SEQ ID NO:1) and *spoIIIJ*-2 (5'-ATG GAT CCT ATG CTC TGA AAT CGC CTG GG-3'; SEQ ID NO:2). The amplified fragment was cleaved with *EcoR*I and *Bam*HI, and ligated into the corresponding sites of pMutin2, resulting in pMutin-Δ*spoIIIJ*. Next, *B. subtilis* Δ*spoIIIJ* was obtained by a single cross-over (Campbell-type) integration of pMutin-Δ*spoIIIJ* into the *spoIIIJ* gene of *B. subtilis* 168, in such a way that the *spoIIIJ* gene was disrupted and the *spoVG-lacZ* reporter gene of pMutin2 was placed under the transcriptional control of the *spoIIIJ* promoter region (*See*, Figure 2). Simultaneously, the *jag* gene located downstream of *spoIIIJ* was placed under the control of the isopropyl-β-D-thiogalacto-pyranoside (IPTG)-dependent P*spac* promoter (*See*, Figure 2).

To construct *B. subtilis* Δ*yqjG*, an internal fragment of the *yqjG* gene was amplified with the primers *yqjG*-3 (5'-TGA AGC TTG CCG GGC TGT TTC ACG G-3'; SEQ ID NO:3) and *yqjG*-2 (5'-ATG GAT CCA TCG TCA TCA TCA CAG GGA AGA TG-3'; SEQ ID NO:4). The amplified fragment was cleaved with *Hin*dIII and *Bam*HI, and ligated into the corresponding sites of pMutin2, resulting in pMutin-Δ *yqjG*. Next, *B. subtilis* Δ*yqjG* was obtained by a single cross-over integration of pMutin-Δ*yqjG* into the *yqjG* gene of *B. subtilis* 168, in such a way that the *yqjG* gene was disrupted and the *spoVG-lacZ* reporter gene of pMutin2 was placed under the transcriptional control of the *yqjG* promoter region (*See*, Figure 2).

To construct *B. subtilis* Δ*yqjG*-l*spoIIIJ*, a fragment comprising the *yqjG* gene and upstream and downstream sequences was first amplified with the primers *yqjG*-1 (5'-GCT TTG GAT TTC TTT TGC CGT CTC-3'; SEQ ID NO:5) and *yqjG*-4 (5'-

GGT TCG TGA GCA TAA AGG GAA GC-3'; SEQ ID NO:6). The amplified fragment was cleaved with *Xba*I and *Kpn*I, and ligated into the corresponding sites of pUK21. Next, the 714 bp *Eco*RI and *Pst*I fragment, containing the 5' sequences of the *yqjG* gene, was replaced with a tetracycline resistance marker resulting in plasmid pUK*yqjG*-Tc. Next, the chromosomal *yqjG* gene of *B. subtilis* 168 was largely deleted by a double cross-over recombination event with linearized pUK*yqjG*-Tc, resulting in *B. subtilis yqjG*-Tc (*See*, Figure 2).

To construct *B. subtilis ΔyqjG-lspoIIIJ*, first a fragment comprising the ribosome binding site, start codon and the 5' region of the *spoIIIJ* gene, but not the *spoIIIJ* promoter(s), was amplified with the primers *spoIIIJ*-3 (5'-GGA ATT CTA GAG TGT AAA GAT TAA TTA TAG GAG GAA ATG TTG-3'; SEQ ID NO:7) and *spoIIIJ*-2. The amplified fragment was cleaved with *Eco*RI and *Bam*HI, and ligated into the corresponding sites of pMutin2, resulting in pMutin-l*spoIIIJ*. Finally, *B. subtilis* Δ *yqjG*-l*spoIIIJ* was obtained by Campbell-type integration of pMutin-l*spoIIIJ* into the *spoIIIJ* gene of *B. subtilis yqjG*-Tc, in such a way that the *spoIIIJ* gene and the downstream *jag* gene were placed under the control of the IPTG-dependent P*spac* promoter, whereas the *spoVG-lacZ* reporter gene of pMutin2 was placed under the transcriptional control of the *spoIIIJ* promoter region (*See*, Figure 2).

B. subtilis ΔyqjG-lspoIIIJ containing a xylose-inducible *secDF-Myc* gene was obtained by transformation of *B. subtilis ΔyqjG-lspoIIIJ* with chromosomal DNA of the xSecDF-Myc strain (Bolhuis *et al.*, 1998). All constructed strains were selected on plates with the proper antibiotics, and checked by PCR analyses for correct integration of plasmids into the chromosome.

EXAMPLE 3
Growth and Maintenance of IPTG-Dependent *B. subtilis* ΔyqjG-lspoIIIJ Strains

The IPTG-dependent strain *B. subtilis ΔyqjG-lspoIIIJ*, and derivatives thereof, were grown in media containing 500 nM IPTG, Em (1 µg/ml) and Tc (6 µg/ml). It should be noted that in the absence of IPTG, such strains sometimes start to grow again after a lag period. This is probably due to the occurrence of a point mutation in the P*spac* promoter, causing constitutive expression of the downstream genes

(Prágai and Harwood, J. Bacteriol., 182:6819-6823 [2000]). To avoid this potential problem, prior to each experiment, individual colonies were replica-plated on plates without IPTG and colonies displaying no growth on the latter plates were used for SpoIIIJ/ YqjG-depletion experiments. To this purpose, *B. subtilis* Δ*yqjG*-l*spoIIIJ* was grown overnight in TY medium supplemented with 500 nM IPTG, Em (1 µg/ml) and Tc (6 µg/ml). Cells were washed in fresh TY medium without IPTG and diluted 1:20 in fresh TY medium without, or with limiting concentrations (50 nM) of IPTG. After 3 hours of growth, cells were harvested. Δ*yqjG*-l*spoIIIJ* strains stop growing after about 2-3 hours in the absence of IPTG (*See,* Figure 3, Panel A).

EXAMPLE 4
Sporulation and Growth of *Bacillus*

In this Example, results obtained regarding growth and sporulation of *B. subtilis* are described.

YqjG is Not Required for Sporulation

As indicated above, a *yqjG* disruption strain was constructed with the integration vector pMutin2 (*See*, Figure 2). The fact that *B. subtilis* Δ*yqjG* could be obtained showed that YqjG, like SpoIIIJ, is not essential for growth (*See*, Figure 3, Panel A). Notably, disruption of the *yqjG* gene did not detectably affect sporulation, while a Δ*spoIIIJ* control strain constructed with pMutin2 was unable to develop viable spores (data not shown). Thus, YqjG has no essential function in the sporulation process, in contrast to SpoIIIJ.

The Presence of Either SpoIIIJ or YqjG is Required for Growth of *B. subtilis*

To determine whether the combined activities of SpoIIIJ and YqjG are required for the viability of *B. subtilis* cells, a conditional (IPTG-dependent) *yqjG-spoIIIJ* double mutant strain was constructed in two steps. First, the largest part of the *yqjG* gene was replaced with a tetracycline resistance marker *via* double cross-over recombination. Second, the *spoIIIJ* gene of the latter strain was placed under the control of the isopropyl-β-D-thiogalacto-pyranoside (IPTG)-inducible P*spac* promoter, present on pMutin2. This was achieved by single cross-over of pMutin2 into the *spoIIIJ* region of the chromosome. (*See*, Figure 2). Notably, the resulting *B.*

subtilis Δ*yqjG*-I*spoIIIJ* strain could be obtained only in the presence of IPTG, indicating that at least the *spoIIIJ* gene had to be transcribed for the growth of this strain. In fact, as indicated in Figure 3, Panel A, about 500 nM IPTG was required for this strain to display unimpaired growth on TY agar plates at 37°C. Upon dilution in fresh TY medium without IPTG, *B. subtilis* Δ*yqjG*-I*spoIIIJ* stopped growing after 2-3 hours of incubation (*See*, Figure 3, Panel B). Similar to the IPTG-dependent growth of this strain on TY plates, the presence of 500 nM IPTG was required to support wild-type growth of *B. subtilis* Δ*yqjG*-I*spoIIIJ* in liquid TY medium at 37°C. In contrast, growth of *B. subtilis* Δ*yqjG*-I*spoIIIJ* in the presence of 50 nM IPTG was significantly reduced as compared to that of the parental strain 168, indicating that SpoIIIJ was synthesized at limiting levels. Importantly, growth of *B. subtilis* Δ*yqjG*-I*spoIIIJ* in the absence of IPTG could be restored by the ectopic expression of the *spoIIIJ* gene, showing that the growth inhibition of this strain is due to SpoIIIJ limitation, and not to polar effects on the expression of the downstream-located *jag* gene. Together with the observation that *spoIIIJ* and *yqjG* single mutants are viable, these findings show that the presence of either SpoIIIJ or YqjG is required for growth of *B. subtilis*. Notably, the IPTG-dependence of *B. subtilis* Δ*yqjG*-I*spoIIIJ* was strongly increased at 15°C (*See*, Figure 3, Panel A). As protein transport *via* the Sec pathways of *B. subtilis* and *E. coli* is intrinsically cold-sensitive (Bolhuis *et al.*, [1998], *supra*; Pogliano and Beckwith, *supra*; and van Wely *et al.*, [1999], *supra*), the observed temperature effect suggests that SpoIIIJ and YqjG have a role in Sec-dependent protein transport.

Maximal spoIIIJ and yqjG Transcription in the Exponential Growth Phase

Previous studies showed that the *spoIIIJ* gene is transcribed during the exponential growth phase and that this transcription is shut down at about the onset of sporulation (See, Errington *et al.*, *supra*). To determine whether the transcription of the *yqjG* gene is regulated in a similar manner, the transcriptional *yqjGlacZ* and *spoIIIJ-lacZ* gene fusions that are present in *B. subtilis* Δ*yqjG* and Δ*spoIIIJ*, respectively (*See*, Figure 2) were used during the development of the present invention. Both strains were grown in TY medium, minimal medium, or sporulation medium, and samples withdrawn at hourly intervals were assayed for β-

galactosidase activity. The results showed that, irrespective of the growth medium, the β-galactosidase levels in both strains reached a maximum in the exponential phase (*See,* Figure 3, Panel C--only the results obtained with cells grown in TY medium are indicated). The β-galactosidase levels were strongly decreased upon the transition (t=0) into the post-exponential growth phase. As the transcription profiles of the *yqjG-lacZ* gene fusion were very similar to those of the *spoIIIJ-lacZ* fusion under all conditions tested, it seems highly unlikely that the lack of effect of a *yqjG* mutation on sporulation is due to differences in the transcription of these genes.

EXAMPLE 5
Protein Secretion

In this Example, results of experiments to assess protein secretion by *B. subtilis* and various mutant strains are described.

SpoIIIJ and YqjG are Required for Efficient Protein Secretion

To evaluate the importance of YqjG and SpoIIIJ function for protein secretion, *B. subtilis* Δ*yqjG*, Δ*spoIIIJ*, and Δ*yqjG*-I*spoIIIJ*, as well as the parental strain 168, were transformed with plasmid pLip2031 for the secretion of the *B. subtilis* lipase LipA (Dartois *et al., supra*), pPSPPhoA5 for the secretion of the alkaline phosphatases PhoA of *E. coli* fused to the prepro-region of the lipase gene from *Staphylococcus hyicus* (Bolhuis *et al.*, [1999b], *supra*), or pKTH10 for the secretion of the a–amylase AmyQ (Palva, *supra*). As no secretion defects were detectable in the single mutant strains (data not shown), attention was focused on the Δ*yqjG*-I*spoIIIJ* double mutant. In order to deplete *B. subtilis* Δ*yqjG*-I*spoIIIJ* of SpoIIIJ, this strain was
grown for three hours in TY medium without IPTG, as described above. As a control, TY medium with 50 nM IPTG (limiting amounts of SpoIIIJ), or 500 nM IPTG (full induction of SpoIIIJ) was used (*See*, Figure 3, Panels A and B). Next, the secretion of LipA, PhoA and AmyQ was analyzed by Western blotting, as described above. As shown in Figure 4, Panel A (lower panels), the levels of LipA, PhoA and AmyQ in the medium of SpoIIIJ-depleted cells of *B. subtilis* Δ*yqjG*-I*spoIIIJ* (no IPTG) were significantly reduced compared to those in the media of the fully induced double mutant (500 nM IPTG), or the parental strain 168. Moreover, SpoIIIJ-

depleted double mutant cells containing pPSPPhoA5 or pKTH10 also contained significantly decreased levels of mature PhoA or AmyQ, respectively (*See*, Figure 4, Panel A). In contrast, the cellular levels of mature LipA, and the precursor forms of LipA and AmyQ were not affected by SpoIIIJ depletion in the absence of YqjG (*See*, Figure 4, Panel A, upper panels). Interestingly, the levels of LipA and PhoA in the media of Δ*yqjG*-l*spoIIIJ* strains that were fully induced with IPTG (500 nM), were higher than those in the media of the parental control strains. The latter finding suggests that overexpression of the *spoIIIJ* gene can result in improved protein secretion in *B. subtilis*.

To investigate the nature of the secretion defect of *B. subtilis* Δ*yqjG*-l*spoIIIJ*, further experiments were performed with AmyQ and an AmyQ variant (AmyQ-PSBT) as described by Bolhuis *et al.* (Bolhuis *et al.*, [1998], *supra*). As shown by pulse-chase labeling experiments, at early chase times (0 and 1 min of chase), the pre-AmyQ synthesis and processing in cells of *B. subtilis* Δ*yqjG*-l*spoIIIJ* depleted of SpoIIIJ (no IPTG) was not significantly different from that observed in cells of this strain in which SpoIIIJ was fully induced (500 nM IPTG; See, Figure 4, Panel B), or the parental strain 168 (data not shown). However, in particular after 5 min of chase, significantly reduced amounts of mature AmyQ were detectable in the Δ*yqjG*-l*spoIIIJ* cells depleted of SpoIIIJ. This suggests that these cells have a defect in post-translocational protein folding rather than a defect in protein translocation. To verify that SpoIIIJ-depleted cells lacking YqjG have no translocation defect, experiments were performed with AmyQ-PSBT, which contains a carboxyl-terminal biotin accepting domain (PSBT) of a transcarboxylase from *Propionibacterium shermannii* (Tjalsma *et al.*, [1998], *supra*). The rationale of this experiment is that pre-AmyQ-PSBT can only be biotinylated by the cytoplasmic biotin ligase when the PSBT domain folds into its native three-dimensional structure prior to translocation. Consequently, biotinylation of pre-AmyQ-PSBT occurs at significantly increased levels when pre-AmyQ-PSBT translocation is slowed-down, for example by the disruption of the *secDF* gene (Bolhuis *et al.* [1998], *supra*). As shown in Figure 4, Panel C, SpoIIIJ-depleted cells lacking YqjG did not accumulate increased amounts of biotinylated pre-AmyQ-PSBT as compared to cells in which SpoIIIJ synthesis was induced with IPTG, or the parental strain 168. In contrast, *B.*

subtilis cells with a disrupted *secDF* gene (positive control) accumulated strongly increased amounts of biotinylated AmyQ-PSBT (*See*, Figure 4, Panel C). Notably, the biotinylated AmyQ-PSBT in the Δ*secDF* cells was found to be present both in the precursor and mature forms. Taken together, these observations support the view that the translocation of pre-AmyQ is not affected in SpoIIIJ-depleted cells lacking YqjG. Instead, SpoIIIJ and YqjG appear to be very important for the post-translocational folding stages in protein secretion.

SpoIIIJ and YqjG are Required for Efficient Post-Translocational Folding and Release of Secretory Proteins

While previous data show that the depletion of YqjG and SpoIIIJ affects a post-translocational step in protein secretion, it does not show whether AmyQ, PhoA and LipA are degraded before or after release into the medium of *B. subtilis.* Therefore, a pulse-chase experiment followed by protein localization was performed as described above, in order to identify the site at which secretory proteins are subject to degradation. As shown in Figure 5, Panel A, the pre-AmyQ synthesis and processing (upper panel) in cells of *B. subtilis* Δ*yqjG*-l*spoIIIJ* depleted of SpoIIIJ (no IPTG), and release of AmyQ into the cell wall (middle panel) were not significantly different from that observed in cells of the parental strain. However, the amount of AmyQ that was released into the medium after 10 min of chase (lower panel), was about three-fold reduced in the Δ*yqjG*-l*spoIIIJ* cells depleted of SpoIIIJ. Furthermore, a specific AmyQ degradation product was only released into the medium of Δ*yqjG*-l*spoIIIJ* cells depleted of SpoIIIJ. Similarly, the pre-pro-PhoA synthesis and processing (*See*, Figure 5, Panel B, upper panel) in cells of *B. subtilis* Δ*yqjG*-l*spoIIIJ* depleted of SpoIIIJ (no IPTG), and release of AmyQ into the cell wall (middle panel) were not significantly different from that observed in cells of the parental strain. It was noted that pro-PhoA is extremely unstable, as at least four degradation products of pro-PhoA were detected in the cell wall and medium fractions (middle and lower panels). However, the levels of these degradation products were significantly reduced in the cell wall of *B. subtilis* Δ*yqjG*-l*spoIIIJ* depleted of SpoIIIJ. Furthermore, the release of these pro-PhoA forms and mature PhoA into the medium of *B. subtilis* Δ*yqjG*-l*spoIIIJ* depleted of SpoIIIJ was found to be reduced compared to the parental strain (about 2-fold; lower panel). Finally, a

specific PhoA degradation product was only released into the medium of Δ*yqjG*-I*spoIIIJ* cells depleted of SpoIIIJ (lower panel). Similarly, the LipA synthesis and processing (See, Figure 5, Panel B, upper panel) in cells of *B. subtilis* Δ*yqjG*-I*spoIIIJ* depleted of SpoIIIJ (no IPTG), and release of AmyQ into the cell wall (middle panel) was not significantly different from that observed in cells of the parental strain. However, the release of LipA into the medium of Δ*yqjG*-I*spoIIIJ* cells depleted of SpoIIIJ was slightly impaired compared to the release of LipA from the cell wall of the parental strain (middle and lower panels). It was noted that under these conditions the LipA secretion defect in cells of *B. subtilis* Δ*yqjG*-I*spoIIIJ* depleted of SpoIIIJ seems to be mild compared to the defect observed in rich medium (*See*, Figure 4, Panel A). Together, these data show that mature AmyQ and (pro-)PhoA are significantly less stable in the membrane-cell wall interface of *B. subtilis* Δ*yqjG*-I*spoIIIJ* depleted of SpoIIIJ. The fact that degradation products are already released into the medium at t=1', indicates that degradation occurs soon after the precursors have left the translocation channel. Thus, these observation indicate that the secretion defects in *B. subtilis* cells depleted of YqjG and SpoIIIJ relate to impaired folding of secretory proteins in the membrane-cell wall interface and subsequent degradation prior to release of the secretory protein into the medium.

EXAMPLE 6

Membrane Protein Stability and Topology

In this Example, results from experiments conducted to assess membrane protein stability and topology are described.

SpoIIIJ and YqjG are of Minor Importance for Membrane Protein Stability and Topology

As shown by Samuelson *et al.* (Samuelson *et al.*, *supra*), the YidC protein of *E. coli* is very important for the correct insertion of various proteins into the inner membrane, but not the export and processing of Sec-dependent pre-proteins. To investigate whether the SpoIIIJ and YqjG functions could be required for membrane protein biogenesis in *B. subtilis*, the cellular levels of the BdbB, BdbC, FtsH, PrsA, SecDF-Myc, SipS, and SPase II proteins in the Δ*yqjG*-I*spoIIIJ* strain were monitored by Western blotting. These proteins were primarily selected because they have

different membrane topologies and different numbers of transmembrane segments (*See*, Figure 6; results are not shown for BdbB/C and SPase II, which have four transmembrane segments and an Nin-Cin topology). Furthermore, these proteins, which are known to be involved in protein secretion by *B. subtilis* (Tjalsma *et al.*, [2000a], *supra*), were selected to investigate whether the secretion defects of SpoIIIJ-depleted cells lacking YqjG might be indirectly caused by the impaired membrane biogenesis of secretion machinery components. Interestingly, the levels of SecDF-Myc, SipS, and PrsA were not detectably affected in SpoIIIJ-depleted cells lacking YqjG that were grown at 37°C (*See*, Figure 6), or 15°C (data not shown). Similarly, the cellular amounts of BdbB, BdbC and SPase II remained unchanged (data not shown). In fact, FtsH was the only protein involved in protein secretion (Deuerling *et al.*, Mol. Microbiol. 23:921-933 [1997]) that was (mildly) affected upon SpoIIIJ depletion in the absence of YqjG. As shown in Figure 6, low amounts of FtsH degradation products (lower panel) accumulated in Δ*yqjG-lspoIIIJ* cells depleted of SpoIIIJ.

The Oxa1p protein of yeast mitochondria was previously shown to be required for the correct assembly of cytochrome c oxidase complexes (Bonnefoy *et al.*, Proc. Natl. Acad. Sci. USA 91:11978-11982 [1994]; and Altemura *et al.*, FEBS Lett., 382:111-115 [1996]), and the specific export of the amino- and carboxyl-termini of pre-Cox II from the mitochondrial matrix to the intermembrane space (Hell *et al.*, [1997], *supra*; Hell *et al.*, [1998], *supra*; and He and Fox, Mol. Biol. Cell., 8:1449-1460 [1997]). Therefore, a possible role of SpoIIIJ and YqjG in the biogenesis of the transmembrane lipoproteins CtaC and QoxA, which are orthologues of Cox II (Bengtsson *et al.*, J. Bacteriol., 181:685-688 [1999]), was investigated during the development of the present invention. Indeed, the results showed that, upon SpoIIIJ depletion of cells lacking YqjG, the cellular levels of CtaC were slightly reduced, while a specific CtaC degradation product accumulated concomitantly (*See*, Figure 6). However, as also shown in Figure 6, under the same conditions, the cellular level and stability of QoxA were not detectably affected.

Previous studies by de Gier *et al.* (de Gier et al., FEBS Lett., 399:307-309 [1996]) have shown that impaired membrane protein insertion does not necessarily affect the total cellular levels of membrane proteins. To determine whether

membrane proteins were properly inserted in SpoIIIJ-depleted cells lacking YqjG, the membrane topology of SecDF-Myc, SipS, PrsA, FtsH, CtaC and QoxA was assessed by protoplasting and subsequent protease mapping assays, as described in Example 1. As shown in Figure 7, the protease accessibility of none of the tested membrane proteins, was detectably affected in Δ*yqjG*-I*spoIIIJ* cells upon SpoIIIJ depletion. The cytoplasmic protein GroEL was not degraded by extracellular trypsin, indicating that lysis of protoplasts did not occur during the assay. It should be noted that the degradation product of FtsH, which accumulates in Δ*yqjG*-I*spoIIIJ* cells depleted of SpoIIIJ (*See*, Figure 6), was also detectable in the parental strain upon protoplasting. Also, the degradation product of CtaC, which accumulates in Δ*yqjG*-I*spoIIIJ* cells depleted of SpoIIIJ (*See*, Figure 6), was not detectable upon protoplasting of these cells.

To examine the kinetics of membrane protein insertion, a pulse-chase/protease mapping experiment was performed with cells of the parental *B. subtilis* 168 strain and Δ*yqjG*I*spoIIIJ* cells upon SpoIIIJ-depletion. As shown in Figure 8, the protease accessibility of SipS, PrsA and QoxA was not detectably affected in Δ*yqjG*-I*spoIIIJ* cells upon SpoIIIJ depletion after a 1-min chase. GroEL was not degraded by extracellular trypsin, indicating that no lysis of protoplasts occurred during this assay. Consistent with the lack of effect on the insertion of PrsA in the membrane, the processing of pre-PrsA by SPase II, as verified by pulse-chase labeling, was not affected in Δ*yqjG*-I*spoIIIJ* cells upon SpoIIIJ-depletion (data not shown). Unfortunately, neither SecDF-Myc, CtaC, nor FtsH were tested in pulse-chase experiments as it was not possible to immunoprecipitate these proteins, most likely due to rapid degradation by endogenous *B. subtilis* proteases.

Taken together, these findings show that the stability of some membrane proteins is affected in cells with limiting amounts of SpoIIIJ and YqjG. However, the stability, topology and insertion kinetics of other membrane proteins and membrane-associated components of the *Bacillus* secretion machinery is not significantly affected under those conditions. Thus, it is contemplated that the defect in the secretion of AmyQ, LipA and PhoA secretion is not caused by impaired membrane assembly of individual protein secretion machinery components.

From the above, it is clear that the present invention provides methods and

compositions for the modulation of Sec-dependent protein secretion. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, protein expression, and/or related fields are intended to be within the scope of the present invention.